

EFFECTS OF ORALLY ADMINISTERED POLYPHENOLS FROM BLACK SOYBEANS SEED COATS ON PREVENTING THE DIET-INDUCED INCREASE OF CHOLESTEROL LEVELS IN HYPERCHOLESTEROLEMIC HAMSTERS

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Received on: 20th May, 2016

Accepted on: 25th July, 2016

The aim of present study is to investigate the hypocholesterolemic effects of orally administered Black soybeans Seed coats Polyphenols (BSP) on cholesterol metabolism in hamsters fed with a hypercholesterolemic diet. Male four-week-old hamsters were divided into 3 groups of 10 hamsters each with similar means of body weights and serum cholesterol concentrations. Hamsters fed with the hypercholesterolemic diet were orally administered BSP or Grape Seed Polyphenols (GSP) at a dose of 100 mg/kg body weight for 30 days. The metabolic parameters of lipids and cholesterol in serum, liver and feces were determined. The BSP, as well as GSP, lowered the concentrations of plasma total and LDL-cholesterol and the concentrations of liver free cholesterol significantly compared with control group ($P < 0.05$). The excretion of cholesterol in feces was increased in BSP and GSP groups compared with control group ($P < 0.05$). The concentrations of lactate, acetate, butyrate and total short chain fatty acids (SCFAs) in BSP group were significantly higher than those in control group ($P < 0.05$). These results indicate that the orally administered BSP improves cholesterol metabolism by promoting the excretion of fecal lipids and increasing colonic SCFAs in hamsters fed with the hypercholesterolemic diet.

Keywords: Black soybeans, Polyphenols, Hypercholesterolemia, Fecal lipids, Short-chain fatty acids, Hamsters

INTRODUCTION

Cholesterol as one of the major components of cell membrane plays an essential role in keeping human healthy by influencing cell membrane integrity and lipid fluidity (Incardona and Eaton, 2000; and Haucke and Paolo, 2007). Cholesterol can be converted to other essential molecules, such as bile acids, vitamin D and sterol hormones (Arnold and Kwiterovich, 2003). However, despite of its well-established physiological functions, high level of blood cholesterol is closely associated with the development of some chronic diseases, such as obesity, atherosclerosis (Fadini *et al.*, 2014; and Gao *et al.*, 2014), diabetes (Tajima

et al., 2014) and cardiovascular diseases (Weingärtner *et al.*, 2011). Recently, many studies have shown the role of dietary polyphenols in the prevention of these cholesterol-related metabolic diseases (Scalbert *et al.*, 2005; and Wang *et al.*, 2014). Several hundreds of different polyphenols have been identified in foods, so that much interest has been focused on the development of alternative medicinal foods rich in dietary polyphenols to improve chronic diseases (Scalbert and Williamson, 2000).

Black soybean (*Glycine max* L.), a type of soybean with a black seed coat, has been used widely as a functional

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food for many years in Asia. The sales of black soybean based foods, including soymilk, tofu and soy sauce, have grown tremendously during the last few years because of increasing consumer awareness of black soybean as a healthy food ingredient. In China, black soybean has been utilized as a traditional herbal medicine for its physiological functions of antioxidant activity (Ignasius *et al.*, 2009), anti-diabetes (Kurimoto *et al.*, 2013), detoxification, and anti-inflammatory processes (Lee *et al.*, 2009). Many studies attribute the bioactivities of black soybean to its color coats, because black soybean seed coat is rich in various polyphenols, such as catechins, anthocyanins, procyanidins and other flavonoids (Zhang *et al.*, 2013). Several studies have identified the two main types of polyphenols in black soybean seed coat as anthocyanins (Lee *et al.*, 2009) and procyanidins (Ito *et al.*, 2013), and revealed their anti-obesity and hypolipidemic effects (Kwon *et al.*, 2007; and Kanamoto *et al.*, 2011). Kwon *et al.* (2007) clarify the hypolipidemic effects of black soybean anthocyanins by determining plasma triglyceride and cholesterol contents, and Kanamoto *et al.* (2011) argue that black soybean seed coat extract prevents obesity and glucose intolerance in high-fat diet-fed mice based on the determination of plasma glucose, insulin, lipids and adipocytokines. However, the hypocholesterolemic mechanism of polyphenols from black soybean seed coat is still not clear.

In the present study, Black soybeans Seed coats Polyphenols (BSP) extracted by acidic ethanol was orally administered into hamsters and its protective effects on the hypercholesterolemic diet-induced increase of cholesterol levels was analyzed. Not only cholesterol level in plasma but also its metabolism in liver and excretion in feces were determined in order to investigate how BSP impact the cholesterol absorption and the excretion of bile acids in hypercholesterolemic hamsters. Grape Seed Polyphenols (GSP) as a well-known bioactive abundant resource of anthocyanins and procyanidins was used as a positive control.

MATERIALS AND METHODS

Materials

BSP was prepared as follows: black soybeans (local variety) were harvested in autumn 2014 in Hebei province of China. The extracts of black soybean hulls obtained with acidic water and ethanol were purified using absorbent resin and powdered by vacuum freeze-drying at 4 °C for 48 h (Kanamoto *et al.*, 2011). The composition and polyphenol

content of BSP were shown in Table 1. GSP were provided by Xi'an SR Bio-Engineering Co. Ltd. (Xi'an, China). The basic chemical compositions of BSP and GSP were determined using the methods of AOAC 984.13, AOAC 996.11, AOAC 945.16, AOAC 942.05, AOAC 985.29, and AOAC 993.29, respectively. The total amounts of polyphenol and flavonoid were determined as described by Qiu *et al.* (2010). The content of procyanidin was calculated by subtracting the amount of epicatechin from the total amount of flavonoid (Kanamoto *et al.*, 2011). The content of anthocyanin was determined by the method described in the study of Xu *et al.* (2007).

Table 1: Chemical Composition of BSP and GSP

Ingredient	BSP (%)	GSP (%)
Proteins	0.48±0.02	ND
Starch	6.83±0.11	2.92±0.31
Lipids	0.10±0.01	ND
Ash	1.37±0.03	0.95±0.03
Total fiber	2.31±0.05	ND
Water-soluble fiber	1.17±0.01	ND
Insoluble fiber	1.14±0.04	ND
Total polyphenols	78.92±2.43	94.22±3.15
Flavonoids	56.41±0.10	81.52±0.10
Anthocyanins	3.88±0.07	8.63±0.07
Procyanidins	49.20±1.25	78.70±1.05

Note: Means and standard errors were determined for triplicate. ND represented the component was not detected.

Animals and Diets

Male four-week-old hamsters (n = 30) were obtained from Vital River Lab Animal Technology Co., Ltd. (Beijing, China). The hamsters were housed individually in stainless steel cages under controlled temperature (23 ± 2 °C), humidity (55 ± 5%) and air flow conditions with a fixed 12 h light–dark cycle. After 3 days acclimation, hamsters were divided into 3 groups, namely, Control, BSP and GSP. The average body weight and serum cholesterol concentrations of the hamsters were similar for each group. All hamsters were fed with a hypercholesterolemic diet. Experimental diets shown in Table 2 were prepared according to the American Institute of Nutrition (AIN)-93G formula. The additions of cholesterol,

Table 2: Diet Composition (g/kg Diet)

Ingredients	(g/kg)
Casein	200
Corn Starch	397
Soybean oil	70
Cellulose	50
Sucrose	38.486
t-Butylhydroquinone	0.014
Choline Bitartrate	2.5
L-Cystine	3
Maltodextrin 10	132
Mineral Mix	35
Vitamin Mix	10
Cholesterol	10
Bile salt	2
Lard	50
Total	1000

Note: The diets were prepared according to the AIN-93G formula with some modifications as described in Materials and Methods.

bile salts and lard were added to standard diet in order to elevate the concentration of serum cholesterol. Hamsters in BSP or GSP group were orally administered BSP or GSP at a dose of 100 mg/kg body weight for 30 days. Same amount of deionized water was orally administered to control group.

This study was carried out according to the P.R. China legislation regarding the use and care of laboratory animals and was approved by the Animal Ethics Committee of Chinese Center for Disease Control and Prevention (Beijing, China).

Preparation of Plasma and Tissue Samples

The plasma of hamsters in each group was collected from the eye veniplex on the 0, 10th and 20th day, respectively. Hamster feces were collected for 3 days before scarification. The hamsters were fasted for 16 h and then sacrificed by the removal of blood from the abdominal aorta. The livers were immediately excised and then washed with ice-cold

physiological saline solution (0.155 mol/l), followed by a fixing in a buffer solution of 10% (v/v) formalin or a immediately freezing at “80 °C in liquid nitrogen for further analysis.

Analysis of Metabolic Parameters in Plasma and Liver

The plasma lipids concentrations were measured using an Automatic Chemistry Analyzer (7020, Hitachi, Tokyo, Japan). The liver lipids extracted by tissue lysate from tissue lipids assay kits were chemically measured using the method as the kits described. The concentrations of liver lipids were measured by a Tissue total cholesterol assay kit, E1015; Tissue free cholesterol assay kit, E1016; and Tissue triglyceride assay kit, E1003-2 (Nanjing Sen Shellfish Gamma Biotechnology Co., Ltd., Nanjing, China).

Analysis of Metabolic Parameters in Feces

The fecal cholesterol and fecal bile acids contents were measured using the Tissue total cholesterol assay kit, E1015 (Applygen Technologies Co., Beijing, China) and Rat Bile Acid ELISA Kit (Nanjing Sen Shellfish Gamma Biotechnology Co., Ltd., Nanjing, China), respectively. The fecal total lipids contents were measured by Soxhlet method. The content of fecal short chain fatty acid (SCFA) was measured using ion chromatography (DIONEX ICS-3000, ThermoFisher Scientific Inc., USA) as described in the study of Tong *et al.* (2014). Lactate, acetate, propionate, and butyrate were used as standards (Sigma-Aldrich Co. LLC. MO, USA).

Histopathological Analysis

Liver tissues fixed in formalin solution for at least 24 h were embedded in paraffin wax and sectioned (5.0 μm thickness) for histopathological evaluation. Liver sections were stained with hematoxylin and eosin (H&E) using a standard protocol, and then analyzed by light microscopy.

Statistical Analysis

The data were expressed as means with standard errors and analyzed by Tukey-Kramer’s multiple comparison post hoc test. Statistical significance was defined as $P < 0.05$. The analysis was carried out with SPSS (Version 12.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Growth Parameters of Hamsters

Some studies indicate the healthy benefit of GSP in grape

seed on lipid and cholesterol metabolism (Adisakwattana *et al.*, 2010; Ngamukote *et al.*, 2011; and Margalefa *et al.*, 2014). GSP shows a hypocholesterolemic effect on high-fat diet-fed rats through an inhibition of cholesterol digestion and absorption, and it inhibits cholesterol micelles formation and promotes bile acid binding (Adisakwattana *et al.*, 2010). Therefore, the present study is to investigate the mechanism of hypocholesterolemic effect of BSP and whether BSP exerts beneficial effects in the similar manner to GSP. The growth parameters of hamsters were not affected by orally administering BSP and GSP (Table 3). The food intake, initial body weight, final body weight, body weight gain, and adipose tissue weight did not differ from each other significantly among the 3 groups ($P > 0.05$). It might be because that the composition of the experimental diet was just to obtain the abnormal cholesterol model not obesity model (Table 2). Hamsters without obesity were much more appropriate to evaluate the hypercholesterolemic effect than the mice model that the high fat diet-induced cholesterol metabolism abnormally.

Plasma Lipids

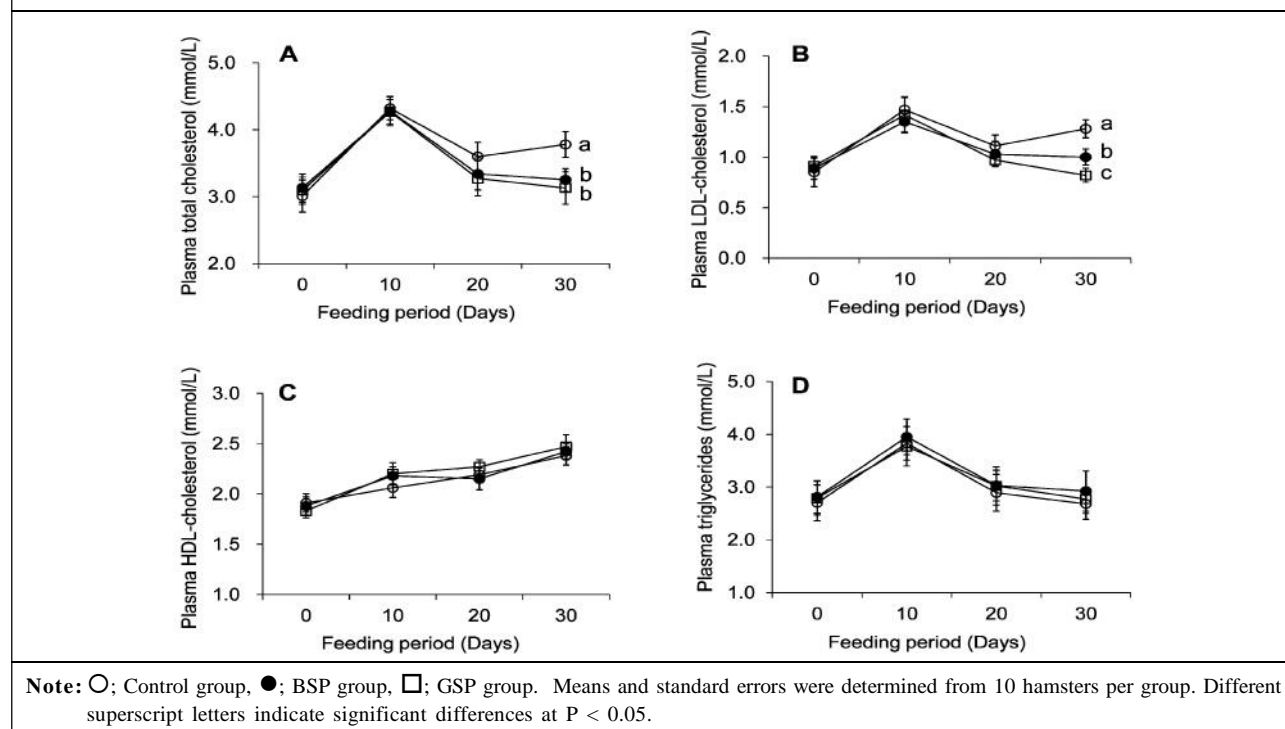
Plasma lipids concentrations at 0, 10, 20, and 30 days were measured and shown in Figure 1. At 30th day, both BSP and

Table 3: Effects of Orally Administrated BSP and GSP on the Growth Parameters of Hamsters Fed a Hypercholesterolemic Diet^a

	Control	BSP	GSP
Food intake (g/day)	6.26±0.22	6.28±0.24	6.36±0.33
Body weight (g)			
Initial	108.4±2.1	108.7±1.8	108.6±1.9
Final	135.5±4.6	136.1±3.8	138.5±3.5
Gain (g)	27.1±3.6	27.4±2.8	29.9±2.5
White adipose tissue (g)			
Total white adipose tissue	13.1±0.91	13.0±0.76	13.3±0.63
Subcutaneous	3.45±0.34	3.57±0.33	3.49±0.28
Mesenteric	1.87±0.23	1.93±0.22	2.01±0.16
Retroperitoneal	1.86±0.19	1.85±0.17	1.76±0.21
Perirenal	2.39±0.24	2.32±0.17	2.49±0.27
Epididymal	3.55±0.25	3.37±0.26	3.52±0.18
Brown adipose tissue (g)	1.23±0.17	1.30±0.14	1.19±0.16

Note: ^a Means and standard errors were determined for 10 hamsters per group

Figure 1: Plasma Total Cholesterol (A), LDL-Cholesterol (B), HDL-Cholesterol (C), and Triglycerides (D) of the Hamsters



GSP prevented the diet-induced increase of the concentrations of plasma total cholesterol and LDL-cholesterol as compared to the control group ($P < 0.05$) (Figures 1A and 1B). The ability of BSP to prevent the diet-induced increase of plasma LDL-cholesterol concentration was slightly weaker than that of GSP at 30th day ($P < 0.05$) (Figure 1B). There was no difference in the concentrations of plasma HDL-cholesterol and triglycerides concentrations among the 3 groups (Figures 1C and 1D). It was observed that dietary BSP, similar to GSP, lowered plasma total and LDL-cholesterol concentrations in hamsters fed with a hypercholesterolemic diet for 30 days, despite the inhibitory effect of BSP on plasma LDL-cholesterol concentrations was slightly weaker than that of GSP. The lowering effect of BSP on plasma total and LDL-cholesterol concentrations was in agreement with previous results reported by Kwon *et al.* (2007) and Kanamoto *et al.* (2011). The difference in plasma LDL-cholesterol between BSP and GSP group might be due to the polyphenols concentration of BSP was lower than that of GSP, together with the different compositions of polyphenols (Table 1). This finding indicated that BSP as an important component in black soybean seed coat was directly responsible for the observed effects on improving cholesterol metabolism, and increased intake of BSP was a feasible therapeutic strategy for prevention and treatment of hyperlipidemia and obesity.

Liver Lipids

With respect to cholesterol metabolism, liver highly coordinates and strictly regulates the biological processes including cholesterol biosynthesis, transformation and transportation, and keeps the maintenance of cholesterol homeostasis (Faust and Kovacs, 2013; and Liu *et al.*, 2016). The liver is an important organ of cholesterol synthesis and decomposition regulated by 3-hydroxy-3-methyl glutaryl-coenzyme A (HMG-CoA) reductase and cholesterol 7- α hydroxylase (CYP7A1) which result in that cholesterol is decomposed into bile acids (Sato *et al.*, 2003). Excretion of lipid, cholesterol and bile acid in feces is a good evidence to illustrate the cholesterol metabolism. Therefore, the metabolic parameters in liver were analyzed in the present study. The concentrations of liver free cholesterol concentration in the BSP and GSP groups were significantly lower than those in the control group ($P < 0.05$). The liver weight, total cholesterol, cholesterol ester, and triglycerides concentrations were not significantly different among the 3 groups. The BSP, as well as GSP, caused the significantly

Table 4: Effects of Orally Administrated BSP and GSP on the Metabolic Parameters in Liver of Hamsters Fed a Hypercholesterolemic Diet^a

	Control	BSP	GSP
Liver weight (g)	5.98±0.26	5.84±0.21	6.07±0.25
Lipids ($\mu\text{mol/g}$)			
Cholesterol			
Total	83.9±7.0	75.5±6.2	80.4±6.7
Free	18.8±1.9	13.9±2.2 ^a	14.5±1.8 ^a
Ester	65.1±6.4	61.6±5.7	65.9±5.3
Triglycerides	23.4±2.1	19.8±1.9	22.3±2.0

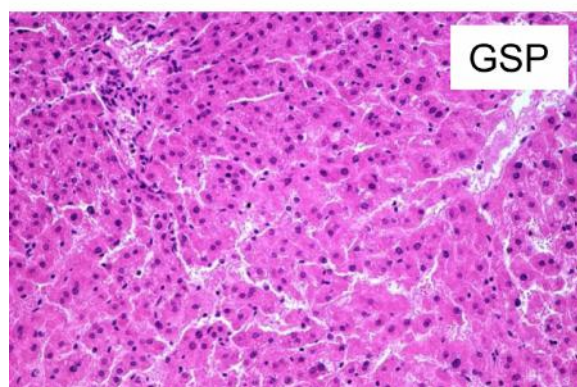
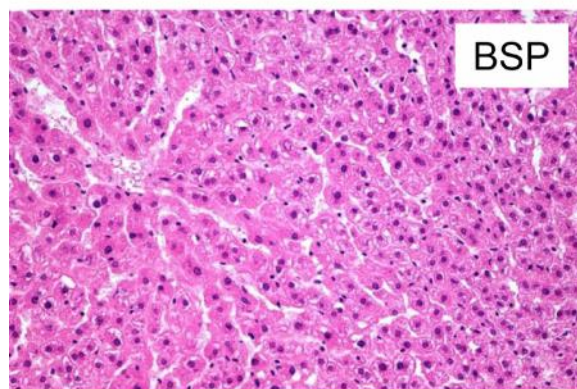
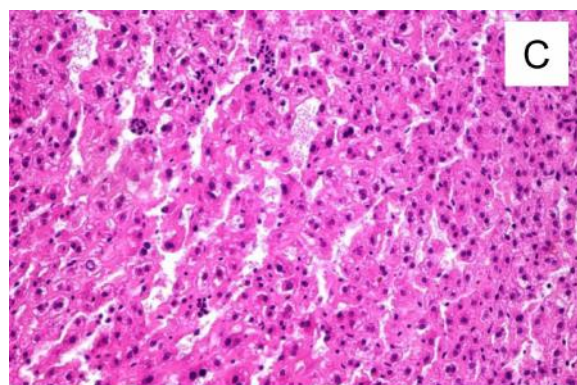
Note: ^a Means and standard errors were determined for 10 hamsters per group. Different superscript letters indicated significant differences at $P < 0.05$.

lower liver free cholesterol concentrations (Table 4), which might be because of the conversion of bile acid. It indicated that BSP could promote the enterohepatic circulation of cholesterol.

Histopathological Analysis

Histopathological changes of hamsters in the 3 groups of 10 hamsters each were shown in Figure 2. The livers of hypercholesterolemic hamsters from the control group showed an accumulation of lipids and hepatic steatosis, in which 8 of them was moderate hepatic steatosis and 2 of them was severe. Moreover, lobular inflammation and ballooned hepatocytes were observed in livers of part hamsters, in which 3 of them was lightly lobular inflammation and 4 of them occurred moderate ballooned hepatocytes. Compared with the control group, BSP and GSP treatment prevented hepatic steatosis, lobular inflammation and ballooned hepatocytes. In the BSP group, 7 of livers were observed lightly hepatic steatosis, and 3 of them were moderate hepatic steatosis; 3 of livers occurred lightly ballooned hepatocytes. The same results as the GSP group, 6 of livers were observed lightly hepatic steatosis; 4 of livers occurred lightly ballooned hepatocytes. Lobular inflammation was not observed in the BSP and GSP groups. It indicated that BSP and GSP prevented hepatic steatosis and lobular inflammation of hamsters induced by hypercholesterolemic diet. This finding was consistent with the reported results that the hypocholesterolemic effect of dietary black soybean was result from the increases of ABCA1 mRNA expression, which increased liver cholesterol

Figure 2: Histopathological Observation of Liver Tissue in the Hamsters, Hematoxylin and Eosin Staining (Original Magnification: $\times 200$), C; Control, BSP; Black Soybeans Seed Coats Polyphenols, GSP; Grape Seed Polyphenols



efflux, thereby prevented non-alcoholic fatty liver disease (Jung and Kim, 2013).

Fecal Lipids

Consequently, the fecal excretion and lipids concentrations were determined in order to clarify the mechanism of the

hypcholesterolemic effect of BSP. Increasing the fecal excretion of bile acids has been hypothesized as a possible mechanism of lowering plasma cholesterol level (Ngamukote et al., 2011). The fecal cholesterol concentration in the BSP group was significantly increased (Table 5). The fecal cholesterol and bile acids excretions were increased in the GSP group compared with the control group ($P < 0.05$), but only fecal cholesterol excretion was increased in the BSP group. In addition, the cholesterol excretion in the GSP group was higher than that in the BSP group ($P < 0.05$). The fecal weight and total lipids concentrations were not significantly different among the 3 groups. This result indicated that the BSP lowered plasma and liver cholesterol concentrations by inhibiting cholesterol absorption in the intestine and increasing the excretion of bile acids. It is noteworthy that the concentration of bile acid in the GSP group was increased significantly while that in the BSP group was tend to be higher even not significant ($P = 0.087$),

Table 5: Effects of Orally Administrated BSP and GSP on the Fecal Parameters of Hamster Fed a Hypercholesterolemic Diet^a

	Control	BSP	GSP
Fecal weight (g/day)	5.54 \pm 0.35	5.33 \pm 0.41	5.86 \pm 0.37
Total lipids (mg/day)	38.1 \pm 1.9	40.7 \pm 2.1	39.6 \pm 1.8
Cholesterol (mg/day)	31.5 \pm 2.5 ^a	38.3 \pm 3.0 ^b	44.9 \pm 2.9 ^c
Bile acids (mg/day)	2.44 \pm 0.06 ^a	2.54 \pm 0.08 ^a	3.10 \pm 0.12 ^b
SCFAs (μ g/day)			
Lactate	247 \pm 23 ^b	431 \pm 23 ^a	264 \pm 18 ^b
Acetate	2312 \pm 85 ^b	3207 \pm 72 ^a	3347 \pm 76 ^a
Propionate	205 \pm 16	196 \pm 20	214 \pm 32
Butyrate	95 \pm 19 ^c	262 \pm 22 ^a	174 \pm 15 ^b
Valerate	ND	51 \pm 4 ^a	22 \pm 8 ^b
Total ^b	2859 \pm 98 ^b	4147 \pm 87 ^a	4021 \pm 92 ^a

Note: ^a Means expressed with standard errors were determined from 10 hamsters per group. ^b The data of fecal SCFAs were the sum of lactate, acetate, propionate, butyrate, and valerate. Different superscript letters indicated significant differences at $P < 0.05$. SCFAs, short-chain fatty acids; ND represented the component was not determined.

which explained the reduced effects of BSP and GSP on liver free cholesterol concentrations (Table 4) and proved the ability of BSP and GSP to promote the conversion from hepatic cholesterol to bile acid. This finding was consistent with the result in the study of Jung and Kim (2013) that black soybean increased the liver bile acids concentration. In addition, the concentrations of cholesterol and bile acids in the GSP group were higher than those in the BSP group. These differences in the inhibition of cholesterol absorption explained our previous result that the effect of GSP on lowering LDL-cholesterol concentrations was better than that of BSP (Figure 1).

Fecal SCFAs Concentrations

SCFAs are very important for preventing the diet-induced increase of cholesterol levels, such as lactate, acetate and butyrate (Drzikova *et al.*, 2005). As shown in Table 5, the concentrations of acetate, butyrate, valerate and total SCFAs in the BSP and GSP groups were higher than those in the control group ($P < 0.05$), and the concentration of lactate in the BSP group was higher than that in the control groups ($P < 0.05$). Moreover, the concentrations of lactate, butyrate, and valerate in the BSP group were slightly higher than those in the GSP group ($P < 0.05$). There was no difference in fecal propionate concentration among the 3 groups. In addition, the valerate was detected in the BSP and GSP groups, but not in the control group. These findings indicated that the hypocholesterolemic effects of BSP were related to the increased colonic SCFAs. Moreover, the concentrations of lactate, butyrate and valerate in the BSP group were higher than those in the GSP group. It might be attributed to their different contents of dietary fiber, because SCFAs are the major end products from the fermentation of dietary fiber by intestinal microflora as reported in wheat (Tong *et al.*, 2014) or oat (Drzikova *et al.*, 2005). In the present study, there was no dietary fiber detected in GSP, but 2.31% of dietary fiber in BSP (Table 1). Therefore, although GSP was more potent than BSP on lowering serum cholesterol and increasing cholesterol excretion, BSP showed better regulation of fecal SCFAs than GSP. The BSP promotion of fecal SCFAs might be attributed to its effect on colonic microflora. Dietary phenolic compounds fermented from polyphenol intake are often transformed by gut microbiota and gut microbial population is modulated by dietary polyphenols in a two-way phenolic-microbiota interaction (Selma *et al.*, 2009). Polyphenols have been reported to influence colonic microflora by modulating microbiota balance, such as polyphenol-rich fruits, wine and green tea

having glycan-degrading enzymes (Lee *et al.*, 2006; and Rastmanesh *et al.*, 2011). The better microbiota balance induced by polyphenols metabolism is a reason why polyphenols exert their weight lowering effect, may thus be a mechanism by which polyphenols increase colonic SCFAs. Furthermore, the potential mechanism of the roles of BSP in promoting the conversion to colonic SCFAs is required to be studied in further study.

CONCLUSION

The present study clearly showed that orally administered BSP prevented the diet-induced increase of plasma total cholesterol and LDL-cholesterol concentrations by promoting fecal bile acids excretion, inhibiting cholesterol absorption in the intestine, and increasing colonic short-chain fatty acids in hamsters fed a hypercholesterolemic diet. BSP is a feasible therapeutic strategy for prevention and treatment of hyperlipidemia and obesity.

ACKNOWLEDGMENT

The Project Sponsored by the Agricultural Science and Technology Innovation Program, Chinese Academy of Agricultural Sciences, and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry (grant number: ([2015]311).

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